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Repressor binding to a dorsal regulatory site traps human eIF4E in a high cap-affinity state

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Eukaryotic translation initiation involves recognition of the 5′ end of cellular mRNA by the cap-binding complex known as eukaryotic initiation factor 4F (eIF4F). Initiation is a key point of regulation in gene expression in response to mechanisms mediated by signal transduction pathways. We have investigated the molecular interactions underlying inhibition of human eIF4E function by regulatable repressors called 4E-binding proteins (4E-BPs). Two essential components of eIF4F are the cap-binding protein eIF4E, and eIF4G, a multi-functional protein that binds both eIF4E and other essential eIFs. We show that the 4E-BPs 1 and 2 block the interaction between eIF4G and eIF4E by competing for binding to a dorsal site on eIF4E. Remarkably, binding of the 4E-BPs at this dorsal site enhances cap-binding via the ventral cap-binding slot, thus trapping eIF4E in inactive complexes with high affinity for capped mRNA. The binding contacts and affinities for the interactions between 4E-BP1/2 and eIF4E are distinct (estimated K_d values of 10^{-8} and 3×10^{-9} for 4E-BP1 and 2, respectively), and the differences in these properties are determined by three amino acids within an otherwise conserved motif. These data provide a quantitative framework for a new molecular model of translational regulation.
Keywords: cap-binding affinity/eIF4E dorsal binding site/eukaryotic translation initiation factors 4E and 4G/gene expression/regulation by 4E-binding proteins

Introduction

Ribosome binding to the 5′ end of cellular mRNAs involves interactions mediated by the cap-binding complex known as eukaryotic initiation factor 4F (eIF4F). The human version of this complex (Figure 1A) comprises the eukaryotic initiation factors eIF4E, eIF4G and eIF4A (Sonenberg, 1996). The relatively small (~25 kDa) cap-binding protein eIF4E tethers the complex to the mRNA cap. The largest eIF4F component (~170 kDa), eIF4G, has binding sites for eIF4E, eIF3, eIF4A and the poly(A)-binding protein PAB (Lamphear *et al*., 1995; Mader *et al*., 1995; Tarun and Sachs, 1996; Morley *et al*., 1997; Imatake *et al*., 1998) (Figure 1A). Mammalian eIF4GI also binds the MAPK-activated protein kinase Mnk1 (Pyronnet *et al*., 1999). eIF3 is believed to tether the 40S ribosomal subunit to eIF4F, whereas binding to PABP may play a role in promoting interaction between the 3′ and 5′ ends of mRNA, while eIF4A (together with eIF4B) catalyses ATP-dependent RNA helicase activity that may promote ribosomal scanning along structured mRNA (Sonenberg, 1996; Sonenberg and Gingras, 1998).

The activity of eIF4F (Figure 1A) in higher cells is subject to tight control (Merrick and Hershey, 1996; Sonenberg and Gingras, 1998). Indeed, deviation from the normal levels of activity can have drastic effects on cellular functions. For example, overexpression of eIF4E leads to cell transformation and changes in cell morphology (De Benedetti and Rhoads, 1990; Lazaris-Karazas *et al*., 1990). On the other hand, the cell needs to regulate eIF4F activity in response to environmental changes and various stimuli, including temperature stress and stimulation by hormones. Elucidation of the mechanism of eIF4F regulation is therefore an important challenge.

The eIF4E–eIF4G interaction is of central importance for cap-dependent initiation, and can be blocked by small regulatory proteins that bind to eIF4E [the 4E-binding proteins, 4E-BPs (Lin *et al*., 1994; Pause *et al*., 1994)]. Inhibition by 4E-BP1 is apparently inversely dependent on the phosphorylation state of this protein, whereby the hypophosphorylated form binds most tightly to eIF4E (Pause *et al*., 1994; Beretta *et al*., 1996; Gingras *et al*., 1998). Cell transformation caused by overexpression of eIF4E is reversed by concomitant overexpression of the 4E-BP1 gene (Rousseau *et al*., 1996). Genetic, biochemical and immunological analyses have indicated that the binding sites for eIF4G of *Saccharomyces cerevisiae* (here referred to as yeIF4G) and the yeIF4E-binding protein p20 overlap within a surface region of yeIF4E (Ptushkina *et al*., 1998). This region maps to the dorsal face relative to the cap-binding slot in the published three-dimensional (3D) structures of the human and yeast cap-binding proteins (Marcotrigiano *et al*., 1997; Matsuo *et al*., 1997). Binding at the dorsal site of yeIF4E seems to induce positive cooperativity in cap-binding at the ventral cap-binding slot (McCarthy, 1998; Ptushkina *et al*., 1998). Yeast p20 is a phosphoprotein (Zanchin and McCarthy, 1995) whose physiological function has yet to be determined.

The molecular mechanism of eIF4E regulation by mammalian 4E-BPs has remained uncertain. Neither the site of action on heIF4E nor the affinity of binding of these repressors has been determined previously. This information is essential for any understanding of the mode of action of the 4E-BPs *in vivo*. Unlike yeast p20 (Altmann *et al*., 1997; De la Cruz *et al*., 1997), the mammalian repressors seem to mediate strong regulation of translation *in vivo* (Sonenberg and Gingras, 1998). This left open the possibility that the 4E-BPs might act via a distinct regu-

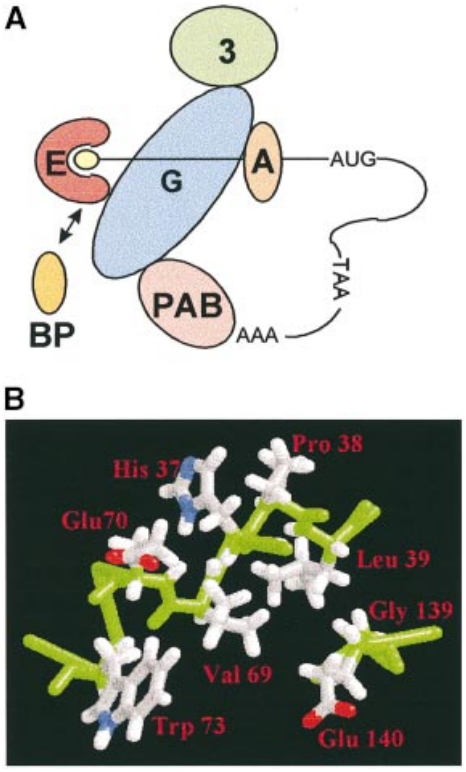

Fig. 1. Repressor binding to the dorsal regulatory site of human eIF4E. (**A**) The cap-binding protein eIF4E (E) is the mRNA cap-binding component of the complex eIF4F. The mammalian complex, which also includes eIF4G (G) and eIF4A (A), is shown interacting with capped mRNA and, via eIF4G, with eIF3 (3) and the poly(A) binding protein (PAB). The eIF4E–eIF4G interaction can be blocked by a 4E-binding protein (BP) in its dephosphorylated state. (**B**) A backbone model based on the known structure of an N-terminally truncated form of murine heIF4E (Marcotrigiano *et al*., 1997) shows the relative positions of amino acids in the dorsal binding site.

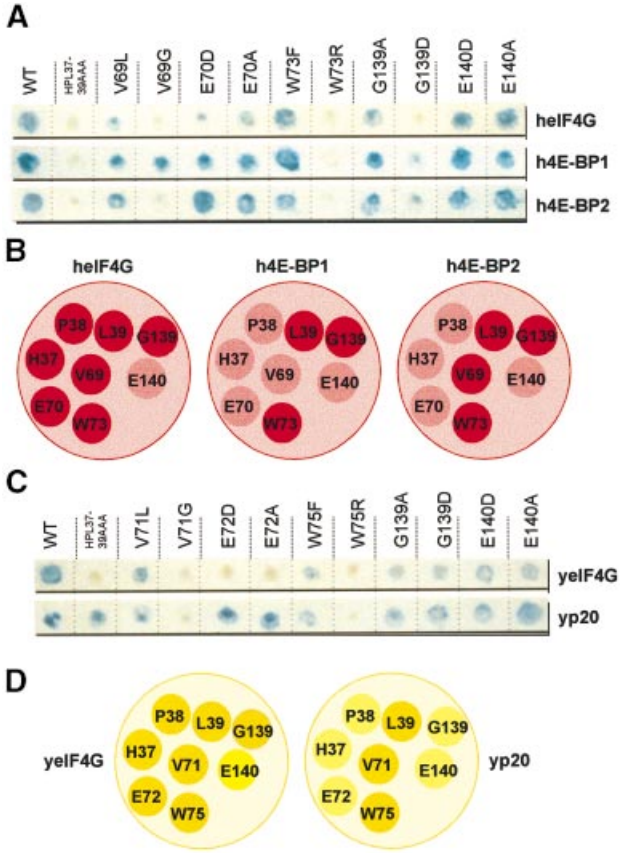

Fig. 2. Two-hybrid analyses were performed to investigate the binding interactions between heIF4E and h4E-BP1, h4E-BP2 and the heIF4E-binding domain of heIF4G, respectively. An interaction is indicated by activation of the *lacZ* (β-galactosidase) reporter gene causing blue colony formation (**A**). The 'interaction footprints' (**B**) represent the two-hybrid data obtained with the eIF4E dorsal site residues (contained in the larger circles). Mutation of some of these residues (marked in dark red) strongly diminishes binding to the respective protein ligands. Further two-hybrid experiments analysed interactions between the dorsal binding region of yeIF4E and yp20 and the yeIF4E-binding domain of yeIF4G, respectively (**C**). The yeIF4E dorsal site residues whose mutation strongly diminishes binding to yp20 or yeIF4G are marked in dark yellow (**D**). Homologous residues between the respective human and yeast binding sites are represented at the same positions in (B) and (D). Not all of the single-site substitution data (for example in region 37–39) upon which the schemes in (B) and (D) are based are shown in (A) and (C) (see text). Each of the interaction profiles was deduced on the basis of three independent sets of experimental data.

latory mechanism to that suggested for yeast (McCarthy, 1998). In the present study, we therefore set out to characterize the qualitative and quantitative parameters underlying the interactions between the 4E-BPs and human eIF4E. Moreover, we questioned whether the 4E-BPs act as modulators of the heIF4E cap-binding function. The results have provided us with a novel model of molecular recognition (mimicry) and an unexpected picture of the mode of action of the human 4E-BPs.

Results

Shared but non-identical binding sites for eIF4G and 4E-BPs on eIF4E

We initiated this study by investigating whether the respective eIF4E binding sites for human eIF4G, 4E-BP1 and 4E-BP2 are in the equivalent surface region of the human cap-binding protein to that of yeast eIF4E characterized in earlier work (Ptushkina *et al.*, 1998). For the sake of both convenience and clarity, we henceforth distinguish human and yeast proteins by using the prefixes h and y, respectively. Mutations were introduced in the dorsal region of heIF4E defined by amino acids H37P38L39 in β-strand 1, V69E70W73 in α-helix 1, and G139E140 in the region between α-helix 2 and β-strand 6 [Figure 1B; see Ptushkina *et al.* (1998) and the structures of the murine and yeast eIF4Es in Marcotrigiano *et al.* (1997) and Matsuo *et al.* (1997)]. The various mutant forms of the gene encoding heIF4E were fused to the Gal4-binding domain in two-hybrid experiments (Fields and Song, 1989) designed to examine interactions between eIF4E and the protein ligands. The genes encoding h4E-BP1 and h4E-BP2, and the gene region encoding the eIF4E-binding domain of heIF4GI [amino acids 507–642 in the eIF4GI sequence reported by Imatake *et al.* (1998)], were fused to the activation domain of Gal4.

Interaction analysis using the two-hybrid system indicated that the respective proteins bind via non-identical molecular contacts to a binding site on the dorsal face of heIF4E (Figure 2A and B). For comparative purposes, we also performed two-hybrid interaction studies between

Table I. Two-hybrid results for h4E-BP1 (MEC→LDR)[a]

Mutation	MEC→LDR	4E-BP1	4E-BP2
Wild-type	8.9	9.7	17.0
L39A	0.9	1.6	1.45
V69G	0	4.3	0.1
W73R	0	0	0
G139D	0.05	0.4	0.2

[a]These are typical data in β-galactosidase units (ONPG units) for the respective two-hybrid pairs.

yeIF4E and, respectively, yeIF4G and yp20 (Figure 2C and D). Figure 2A and C shows typical changes in colony colouring in two-hybrid plate assays for β-galactosidase obtained with the respective eIF4E mutants. We also performed liquid β-galactosidase assays with the same set of mutant strains. Representative data are shown in Table I. These data illustrate the quantitative effects of changes in particular amino acids on the binding of three forms of 4E-BP. The pictorial representations of the two-hybrid data indicate the identities of amino acids whose mutation strongly reduces binding between eIF4E and the protein ligands (Figure 2B and D). These amino acids were identified on the basis of changes in β-galactosidase activity relative to the value obtained with the corresponding wild-type eIF4E. Where a mutation at a given site caused at least a 3-fold decrease in β-galactosidase activity for the interaction with a given protein ligand relative to the other protein ligands, this was recognized as indicative of a differential binding role. Such residues are marked here in dark red (or dark yellow) for the ligand whose binding they differentially affect.

While these profiles are not equivalent to physical maps of the molecular contacts, the differences between them are indicators of at least quantitative differences in binding interactions. Overall, these results tell us that h4E-BP1 and h4E-BP2 compete with heIF4G for the same site on heIF4E, but via molecular contacts that differ with respect to certain residues in the dorsal site. One striking difference is between the evident role of V69 in binding to heIF4G and h4E-BP2 and its apparent lack of significance for h4E-BP1 binding. Not all of the experimental data are shown in Figure 2A. The 37HPL39 region was also analysed using single-site alanine substitutions at all three positions, and the results from these experiments contributed to the schemes shown in Figure 2B.

Experiments performed using yeIF4E and the corresponding yeast protein ligands suggest that yp20 binds via only some of the contacts used by yeIF4G (Figure 2C and D). The deduced contact profiles (Figure 2B and D) show that there are striking parallels between the ways that heIF4E binds to heIF4G and the h4E-BPs and the corresponding patterns of yeIF4E binding to yeIF4G and yp20. It should be noted that the yeIF4E mutations E72D and G139A were reported previously to manifest temperature-sensitive phenotypes (Ptushkina *et al.*, 1998). The two-hybrid experiments described here were performed at 30°C, at which temperature there is either complete (E72D) or partial (G139A) disruption of eIF4G-binding (Figure 2C and D). The comparative two-hybrid data from yeast are also important because they are fully consistent with the earlier analyses of the yeIF4E dorsal binding site that were performed using independent methods (Ptushkina *et al.*, 1998), and therefore confirm the validity of the experimental approach.

Cooperative modulation of cap-binding

Earlier work has indicated that the dorsal binding region of yeIF4E mediates cooperative modulation of cap-binding by yeIF4G (Ptushkina *et al.*, 1998). This raises the question of whether the tightly binding h4E-BPs also act as modifiers of cap-binding via the equivalent site on heIF4E. In order to address this question, we generated recombinant poly(His)-tagged 4E-BPs using *Escherichia coli* as host (Figure 3A). These proteins were found to repress 5′-end-dependent translation, but not IRES-dependent translation, in rabbit reticulocyte lysates (data not shown), thus confirming that they are fully active. The same proteins were then used to examine the ability of heIF4E to bind to m^7GTP–Sepharose as a function of the binding of protein ligands (Figure 3B).

In order to obtain semi-quantitative estimates of the relative amounts of heIF4E captured on the affinity matrix in the presence and absence of protein ligands, we used an image analyser to determine the intensities of the bands in the respective lanes of the silver-stained gels (Figure 3C). The amount of elutable eIF4E captured by the cap-analogue matrix was clearly increased in the presence of either h4E-BP1 or h4E-BP2 compared with the result obtained with heIF4E alone. This effect was much weaker with the p20 protein from yeast, which is not capable of strong binding to the dorsal binding site of heIF4E (compare Figure 4B).

A novel technique for studying RNA–protein interactions

We also examined the phenomenon of stabilization of the cap-bound complex using the surface plasmon resonance (SPR; Fisher and Fivash, 1994) technique to follow the release kinetics for heIF4E bound to a capped and biotinylated RNA 19mer that had been coupled to streptavidin-coated SPR chips (Figure 3D). In optimizing this technique, we observed that RNA oligomers of a similar size with multiple biotin-U insertions were unable to bind heIF4E tightly (data not shown). This was presumably because of steric hindrance by the bulky biotin moieties. We concluded from this and other experiments that this length of RNA allowed the cap to be fully accessible to heIF4E provided that the oligomer was tethered by a single biotin-U placed at its 3′ end.

The stability of binding of heIF4E to the capped RNA was enhanced in the presence of h4E-BP2, resulting in a greatly reduced off-rate from the RNA bound to the chip (Figure 3E). 4E-BP1 was also found to stabilize the heIF4E–cap complex, albeit to a lesser extent (data not shown). Binding of heIF4E was clearly specific for capped RNA, since binding experiments using equivalent amounts of uncapped RNA on the chip yielded signals that were $<5\%$ of the equivalent signals that were obtained with capped RNA (Figure 3F). Moreover, 4E-BP2 alone showed almost undetectable binding to capped RNA in the absence of heIF4E (Figure 3F). Overall, this confirms that the binding of a 4E-BP to the dorsal binding site stabilizes the interaction between heIF4E and the mRNA 5′ cap.

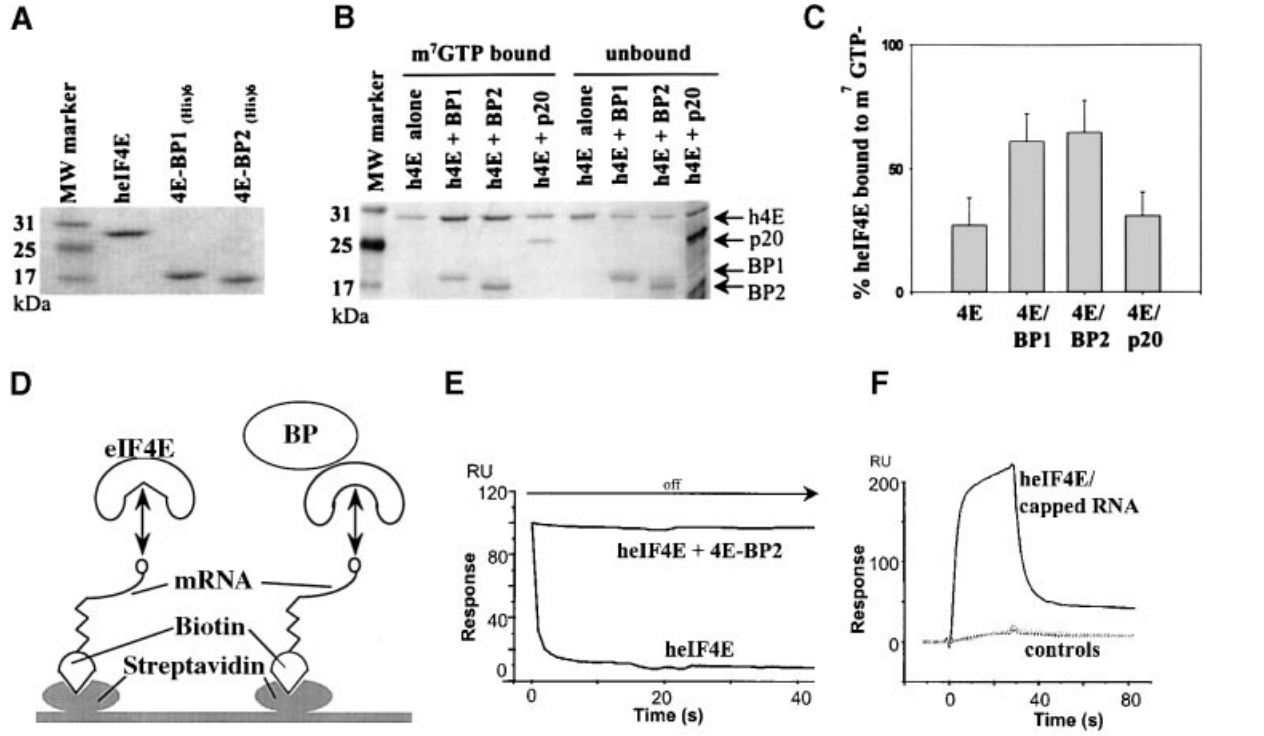

Fig. 3. 4E-BP binding to heIF4E enhances cap affinity. (**A**) Highly pure heIF4E and N-terminally poly(His)-tagged versions of 4E-BP1 and 4E-BP2 were isolated from recombinant *E.coli* strains. The SDS–PAGE gel shows 3 μg of the respective proteins electrophoresed next to protein mass markers after Coomassie Blue staining. (**B**) heIF4E alone or heIF4E plus 4E-BP1, 4E-BP2 or p20 were incubated with m^7GTP–Sepharose affinity resin. After washing with GDP buffer, the eluted proteins were analysed using SDS–PAGE (lanes 2–5). Silver staining was allowed to develop until the weakest bands were just visible. Unbound fractions (i.e. of the material that ran through the column) were loaded in lanes 6–9. (**C**) Quantitation of silver-stained heIF4E band intensities. The values are indicative of the respective amounts (% of total loaded) of heIF4E retained on the affinity resin. Averages of four independent experiments are shown. (**D**) A capped oligoribonucleotide with a 3′-proximal biotinylated U was coupled to a streptavidin-coated SPR chip. (**E**) The kinetics of eIF4E release from the capped RNA in the presence and absence of a 4E-BP are compared. (**F**) A further experiment revealed the on- and off-kinetics for binding of non-complexed (apo–) heIF4E to capped RNA ('heIF4E/capped RNA'). The corresponding curves for heIF4E binding to uncapped RNA and for 4E-BP2 binding to capped and uncapped RNA all approximated to the same baseline ('controls').

Quantitative parameters of eIF4E interactions

Next we proceeded to investigate the interaction between the h4E-BPs and heIF4E that causes the enhancement of cap-binding by the cap-binding protein. Estimates of the relative binding affinities of the h4E-BPs for heIF4E were made using SPR (Figure 4). Poly-(His)-tagged recombinant proteins (h4E-BP1, h4E-BP2 or yp20) were immobilized on nickel-coated chips. Recombinant eIF4E proteins were allowed to interact with the coupled chips, yielding an 'on' phase. Bound eIF4E was then released into buffer lacking added free eIF4E ('off' phase). The results provide estimates of the relative dissociation constants for binding to heIF4E of $1.0 \pm 0.4\times10^{-8}$ and $3.0 \pm 1.5\times10^{-9}$ for h4E-BP1 and h4E-BP2, respectively (Figure 4B). These high binding affinities for heIF4E are specific to the h4E-BPs; yp20 binds poorly, if at all, to heIF4E (Figure 4B). In contrast, yp20, rather than the h4E-BPs, binds tightly to yeIF4E (compare Figure 4A and B). In conclusion, the h4E-BPs both bind specifically to the human version of eIF4E, but they show distinct binding characteristics.

4E-BP1 and 4E-BP2 have distinct binding characteristics

In a previous report (Hughes *et al*., 1999), we described how the repressor function of 4E-BP1 can be studied in *S.cerevisiae*. Translation in this yeast becomes sensitive

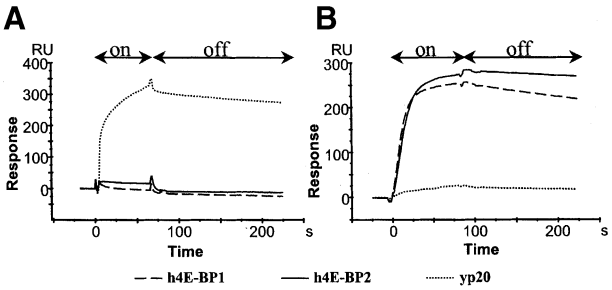

Fig. 4. Binding and release phases for protein ligands of eIF4E. yeIF4E shows rapid binding and slow release only with p20, but not with either of the 4E-BPs (**A**), while heIF4E shows the inverse relationship (**B**). The protein ligands (h4E-BP1, h4E-BP2 and yp20) were bound as C-terminally poly-(His)-tagged proteins to nickel-coated chips and either yeIF4E (A) or heIF4E (B) were passed over the prepared chips.

to the induced synthesis of h4E-BP1 tagged with the FLAG peptide if the endogenous yeIF4E is replaced by heIF4E in an appropriate disruption strain (Hughes *et al*., 1999). We now used this yeast system to examine the inhibitory effect of h4E-BP2 (Figure 5A). The inducible fusion promoter P_{GPF} (Oliveira *et al*., 1993) was used to support synthesis of the h4E-BP genes (in galactose medium; Figure 5A). Synthesis of h4E-BP2, like that of FLAG-tagged 4E-BP1, caused strong inhibition of growth in the yeast strain carrying heIF4E. Western blotting using

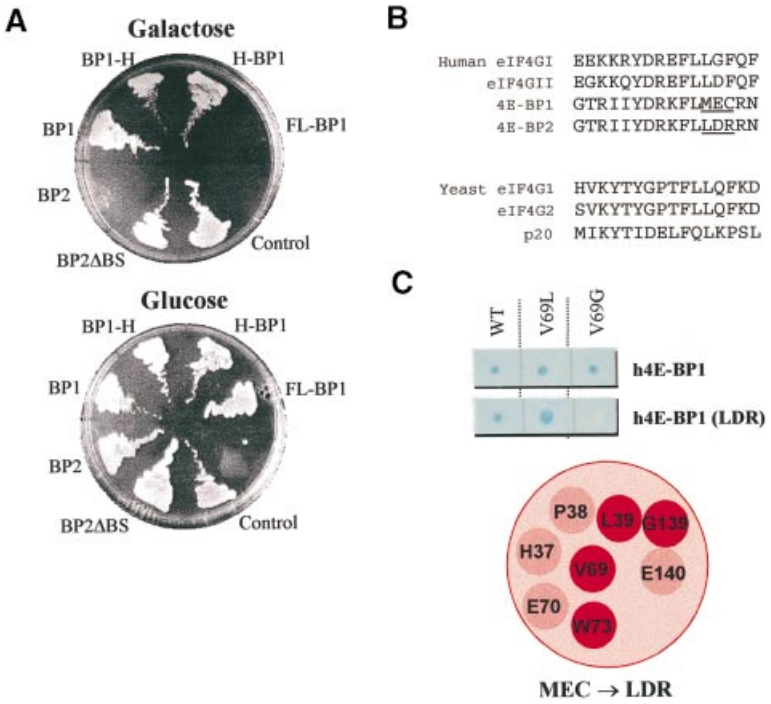

Fig. 5. eIF4E-binding motifs of eIF4G and repressor proteins in man and yeast. (**A**) The plate growth assay (performed at 15°C) shows the effects of various versions of 4E-BP on the growth of a *S.cerevisiae* strain dependent on heIF4E for translational initiation. The respective transformants contained the heIF4E gene under the control of the P_{TEF1} promoter plus the following versions of the 4E-BPs behind the P_{GPF} promoter: non-tagged h4E-BP1 (BP1) or h4E-BP2 (BP2), h4E-BP1 poly(His)-tagged at the N-terminus (H-BP1) or C-terminus (BP1-H), or h4E-BP1 FLAG-tagged at the N-terminus (FL-BP1). A further strain contained the heIF4E plasmid plus the P_{GPF} expression plasmid lacking a 4E-BP gene (Control). (**B**) The human and yeast eIF4G proteins and the h4E-BPs share a motif featuring a generally conserved pattern of charged, aliphatic and aromatic side chains (Mader *et al*., 1995; Altmann *et al*., 1997). A more distantly related version of this motif is present in the N-terminal region of yp20. Comparison of the 4E-BP motifs reveals the substitution of LDR (underlined) in 4E-BP2 for MEC in 4E-BP1. (**C**) Two-hybrid analysis was perfomed using the MEC→LDR mutant form of 4E-BP1. The results are presented in the form of an 'interaction footprint' of 4E-BP2 (compare with Figure 2). Dark red indicates positions at which mutations diminish binding between heIF4E and the MEC→LDR mutant form of 4E-BP1. The small insert shows the key change in the role of V69.

polyclonal antibodies raised against the respective 4E-BPs revealed that the intact repressor proteins were barely detectable (data not shown), suggesting that these proteins are inherently unstable in yeast. Consistent with this, we observed that the growth-inhibition phenotypes were stronger at relatively low growth temperatures (the plates shown in Figure 5A were cultured at 15°C), and were also sensitive to the presence and type of the terminal tag sequence. Thus, synthesis of the FLAG-derivative of h4E-BP1 had a greater repressive effect than poly(His)-tagged or non-tagged h4E-BP1 (Figure 5A), most likely due to a stabilizing influence of the FLAG tag. h4E-BP2 was evidently effective in the absence of a tag sequence. A mutant form of h4E-BP2 (BP2ΔBS) that lacked nine amino acids of the binding motif (Figure 5B; Mader *et al*., 1995) was not able to suppress growth. We conclude that repression is successful in the yeast model system at low ratios of 4E-BPs to heIF4E. Moreover, these results are consistent with the notion that h4E-BP2 is a highly effective repressor of heIF4E function at physiologically relevant concentrations in the cell.

The next question to be addressed was how the distinct specificities of the h4E-BPs are determined at the molecular level. Examination of the respective conserved motifs of these proteins reveals the existence of a three amino acid difference between h4E-BP1 and h4E-BP2 (Figure 5B). Further two-hybrid analyses were performed using a mutant form of h4E-BP1 in which the MEC sub-motif was modified to LDR, thus creating the motif of h4E-BP2. The resulting mutant form of 4E-BP1 was found to have acquired the binding specificity of h4E-BP2 (Figure 5C; Table I). Moreover, the MEC→LDR mutant 4E-BP1 was found to have an increased affinity for heIF4E relative to wild-type 4E-BP1 ($K_d = 4.6 \pm 0.1 \times 10^{-9}$). The 4E-BP binding motif is therefore interchangeable and constitutes a major determinant of specificity.

Discussion

We have characterized a regulatory site on heIF4E that is the target of tight binding by the h4E-BPs. The high binding affinities of the 4E-BPs explain how these two repressors can act as very effective inhibitors of translation when they are in their active (low phosphorylation) states. They are very close to the affinity that we have estimated previously for the interaction between yeIF4E and yeIF4G (Ptushkina *et al.*, 1998), and we conclude on the basis of analogy that the 4E-BPs compete with a similar binding affinity between heIF4E and heIF4G. Another aspect of this regulatory phenomenon to be noted is that the 4E-BPs are effectively acting by at least partially mimicking the binding characteristics of heIF4G. Structural studies on the complex between eIF4E and eIF4G are now needed to determine precisely to what extent molecular mimicry underlies the specificity of the 4E-BPs for the heIF4E dorsal site. It will be particularly important to compare

the whole eIF4E-binding domain of eIF4G with the complete 4E-BP proteins in these studies (as used here; see, for example, Figure 2) in order to ensure that the binding surfaces assume their normal folds (although see later).

The deduced interaction profiles for the two h4E-BPs are slightly different, and possibly less extensive, than that of heIF4G. Moreover, h4E-BP1 and h4E-BP2 manifest distinct binding characteristics that are dictated by three amino acid identities within the eIF4E-binding motif. These distinct binding characteristics suggest that there are differences in the functions of the respective 4E-BPs. It is therefore of interest, for example, that the activities of 4E-BP1 and 4E-BP2 may be subject to distinct modulatory pathways in the course of human myeloid cell differentiation (Grolleau *et al*., 1999). Our work also demonstrates the role of the 16-amino acid motif (Figure 5B) in exercising fine control over the binding characteristics of the 4E-BPs. This motif includes a number of positively charged amino acids, suggesting that electrostatic interactions may play an important role in determining the specificity and affinity of the binding between the 4E-BPs and the heIF4E dorsal binding site, which contains at least one acidic residue that participates in binding (Figure 1B). Indeed, the mutation of MEC in the motif of h4E-BP1 to LDR (h4E-BP2) adds an additional positive charge (R) which may contribute to the increased binding affinity of h4E-BP2 for heIF4E. Possibly of equal significance is the fact that h4E-BP2, like heIF4G, has an L at the first position in this submotif (Figure 5B). This may explain why these two proteins have a stronger interaction with V69 in the heIF4E dorsal binding site compared with h4E-BP1 (Figure 5C).

Since we have used recombinant forms of the h4E-BPs that are non-phosphorylated, our measurements are likely to have defined the maximum affinities that can be achieved between these regulatory proteins and heIF4E. Phosphorylation at multiple sites within the 4E-BP sequences is likely to decrease the affinities for heIF4E (Gingras *et al*., 1998). Given that our work indicates that the dorsal region of heIF4E depicted in Figure 1B is the major site of 4E-BP binding, it is evident that the acidic side chains in this region will provide a negative surface charge. This would be expected to repulse the binding face of the 4E-BPs if these actually bear the sites of phosphorylation, thus suggesting that the phosphorylation sites that regulate 4E-BP binding might need to be spatially close to the binding motif on the 4E-BPs. The rat 4E-BP, called PHAS-I, has been found to have five phosphorylation sites spread over the region 36–82 in the 117 amino acid sequence (Fadden *et al*., 1999). For these to be clustered together on one binding face would require the presence of a folded structure. However, a recent NMR study (Fletcher *et al*., 1998) has indicated that the 4E-BPs have no defined structure in solution. Perhaps, therefore, it will turn out to be the overall negative charge delocalized over a large region of each type of 4E-BP molecule that determines binding affinity rather than localized charge effects associated with specific phosphorylation events. Future work will address this challenging question by examining the properties of various derivatives of the 4E-BPs in different phosphorylation states and/or in differently charged states.

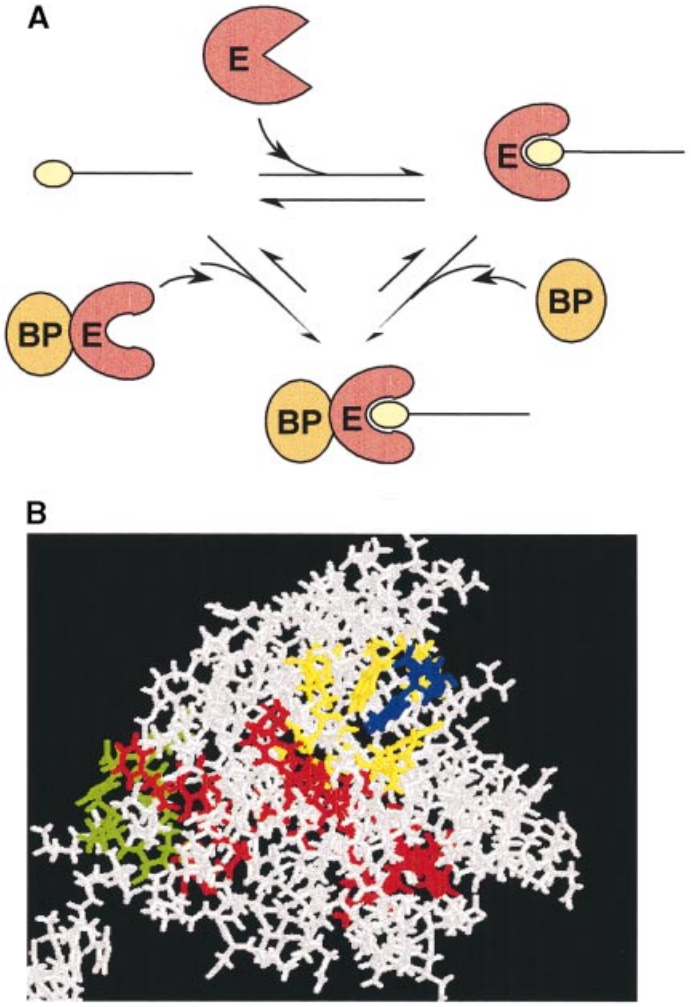

Fig. 6. (**A**) The binding of a 4E-BP shifts the heIF4E population into a form with high affinity for the cap. Both types of 4E-BP can compete with heIF4G for binding to heIF4E due to their comparatively high affinities for a shared dorsal binding site, although 4E-BP2 binds more strongly than 4E-BP1. (**B**) There is a remarkable level of conservation in the 'allostery tract'; the following amino acids (marked in red) are invariant in the eIF4E or (iso)eIF4E proteins of the organisms *S.cerevisiae*, *Schizosaccharomyces pombe*, rat, rabbit, man, mouse, *Drosophila melanogaster*, *Xenopus laevis*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Triticum aestivum* and *Oryza sativa*: W43, W46 (β-strand 1); F66 (β-strand 2); F94 (β-strand 3); P100; G110, G111, W113 (β-strand 4); W130, I138, E140 (α-helix 2); G151 (β-strand 5); I178, G179 (α-helix 3), H200 (α-helix 4) (numbering according to the mammalian eIF4E sequences). This tract runs through the molecule from the dorsal binding site [green; H37, P38, L39 (β-strand 1); V69, E70, W73 (α-helix 1); G139 (α-helix 2)] to the cap-binding slot [yellow; F48 (β-strand 1); W56; L60 (β-strand 2); D90 (β-strand 3); W102, E103; R157 (β-strand 5); W166 (β-strand 6)], which is shown binding to a cap analogue structure (blue).

We have identified a key mechanistic feature of h4E-BP-dependent translational repression: remarkably, the h4E-BPs induce a positive cooperative enhancement of the heIF4E–cap interaction. Earlier cross-linking studies also provided indirect evidence that the cap-affinity of heIF4E is increased upon complex formation with heIF4G (Haghighat and Sonenberg, 1997). While we have concluded previously that in yeast, heterotropic cooperativity may make a positive contribution to the translation initiation cycle supported by the eIF4E–eIF4G complex (Ptushkina *et al*., 1998), the binding of 4E-BPs is expected

to trap eIF4E in an inactive complex with high affinity for capped mRNA that cannot interact with eIF4G (Figure 6A). Thus, both the normal translation initiation cycle and repressor-dependent inhibition involve protein–protein interactions of surprisingly high affinity as well as marked allosteric modulation of eIF4E activity. An important consequence of the formation of a stable complex between heIF4E and a h4E-BP will be that the pool of heIF4G available for participation in non-cap-dependent translation initiation will be increased. Thus, 4E-BP binding may indirectly enhance the capacity of eIF4G to support cap-independent translation *in vivo*.

There is also a remarkable feature of the 3D structure of all types of eIF4E protein studied so far that suggests the existence of a conserved mechanism for the heterotropic cooperativity effect described originally for yeIF4E with yeIF4G (Ptushkina *et al.*, 1998) and now for heIF4E with other protein ligands. We have observed that a tract of highly conserved amino acids stretches from the dorsal binding site through the centre of eIF4E to the cap-binding slot (Figure 6B). Fifteen of these amino acids are fully conserved in 15 different eIF4E protein sequences. We propose that this 'allostery tract' assumes a structure that is essential for the operation of the coupling between the two sites.

In conclusion, this study provides a new mechanistic and quantitative framework for understanding the mode of action of the h4E-BPs, generating testable predictions that can be examined in future work. It shows that the regulation of mammalian translation via the 4E-BPs involves specific recognition by complementary binding sites on the repressor proteins and heIF4E. Recognition of the dorsal binding site of heIF4E, which seems to involve a form of molecular mimicry on the part of the 4E-BPs, induces an allosteric shift that increases cap affinity. We have already proposed that this principle of heterotropic cooperativity could underlie the cycling of the eukaryotic preinitiation complex (Ptushkina *et al.*, 1998). The current work indicates that the cooperativity effect in eIF4E can be induced by a number of protein ligands. Moreover, it is a conserved feature of both higher and lower eukaryotes that may be linked to the presence of a highly conserved 'allostery tract' shared by all of the known eIF4E proteins. Further experiments will need to focus on how this special structural feature allows eIF4E to change its cap-binding affinity in response to binding by its protein ligands, and on the potential physiological significance of this phenomenon.

Materials and methods

Plasmids and protein purification

Mutations were introduced into the *S.cerevisiae* and human eIF4E genes using PCR site-directed mutagenesis (Mikaelian and Sergeant, 1992). For expression of the genes encoding heIF4E, h4E-BP1, h4E-BP2, yeIF4E and yp20 in *E.coli*, the respective genes were subcloned as *Nde*I–*Bam*HI fragments into the vector pCYTEXP1 (Belev *et al.*, 1991). The 4E-BP1, 4E-BP2 and p20 proteins were tagged by introducing an additional sequence encoding six histidine residues. Proteins were synthesized and purified according to the methods described previously (Ptushkina *et al.*, 1998; Hughes *et al.*, 1999). In preparation for two-hybrid assays, the mutant forms of the eIF4E genes were subcloned as *Nde*I–*Bam*HI fragments into the polylinker region of the pGBT9 vector (Clontech) containing the DNA-binding domain of the Gal4 translational activator. The gene sequences encoding yp20, h4E-BP1, h4E-BP2 and the eIF4E-binding domain of heIF4G (h4G-4EBD) were cloned as *Nde*I–*Bam*HI fragments into the polylinker region of the pGAD424 vector (Clontech) bearing the Gal4-activation domain.

Analytical m^7GTP–Sepharose chromatography

In order to determine the relative cap-affinity of eIF4E in the presence of the different binding partners 4E-BP1, 4E-BP2 and p20, 20 μg of eIF4E and each binding partner were incubated together in buffer A (20 mM HEPES pH 7.4, 100 mM KCl, 2 mM $MgCl_2$ and 2 mM DTT) at 4°C for 10 min, then 30 μl of m^7GTP–Sepharose resin were added and the total volume was made up to 300 μl with further buffer A. Incubation was continued at 4°C for 2 h with moderate shaking. The resin was then washed twice with 1 ml of buffer A and bound proteins were eluted with 80 μl of 0.1 mM m^7GDP in buffer A. Eluted fractions (10–20 μl) were analysed on 15% SDS–polyacrylamide gels, and the proteins were rendered visible by silver staining.

SPR assays

All SPR assays were performed using a BIAcore 3000. The Sensorchip NTA (Biacore) was used for immobilizing the His_6-tagged proteins, and human or yeast eIF4E was injected over the chip. Each immobilization cycle consisted of a 20 μl injection of 500 nM $NiCl_2$ in eluent buffer [10 mM HEPES pH 7.5, 100 mM KCl, 50 μM EDTA and 0.005% surfactant p20 (Biacore)], 50 μl of His_6-tagged protein in eluent buffer [resulting in ~250 resonance units (RU) of immobilized protein] and 40 μl of eIF4E in buffer A in a concentration range between 100 and 500 nM. The chip was regenerated after each cycle with 350 mM EDTA in eluent buffer. All measurements were performed at a flow rate of 30 μl/min at 25°C. The resulting sensorgrammes were evaluated using the BIA Evaluation software package. The response from Ni-coated chips without immobilized protein was subtracted from the response obtained with the protein-coated chips. The resulting curves were analysed using local fittings for Langmuir binding and were averaged for each protein. For analysis of eIF4E binding to RNA, an RNA molecule with the sequence 5′-GACACCAACAACAACAUCA-3′ was generated by *in vitro* transcription from oligonucleotides containing the T7 promotor sequence. Transcription was performed using rATP, rCTP, rGTP and Biotin-21-UTP (Clontech) for the transcription of uncapped RNA, or with m^7GpppG (New England Biolabs) substituted for rGTP in the case of capped RNA. The transcribed RNA was purified by means of polyacrylamide gel electrophoresis, phenol extraction and subsequent ethanol precipitation. The RNAs were immobilized on streptavidin-coated chips (Sensorchip SA, BIAcore) after denaturation for 5 min at 96°C followed by cooling for 1 min on ice, generating a signal of 150 RU. The binding of eIF4E was performed in 20 mM HEPES pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 100 mg/l *E.coli* tRNA. eIF4E was injected at a concentration of 2 μM and a flow rate of 30 μl/min for 1 min. This was followed by monitoring of eIF4E-release from the RNA layer. After each measurement cycle the RNA layer was regenerated using injections of 30 μl each of 0.1 mM m^7GDP in binding buffer and 2 M KCl. Further details of this new procedure to analyse RNA–protein interactions are given in T.Von der Haar and J.E.G.McCarthy (paper submitted).

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